Comm.
Dr. Gardner
Dr. Huebner
Dr. Sommers

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THE COUNCIL FOR TOBACCO RESEARCH—U.S.A., INC.
110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

JAN 2 2 1973

Application For Renewal of Research Grant
(Use extra pages as needed)

First Renewal ☒ Second Renewal ☐ Date: January 22, 1973

1. Principal Investigator (give title and degrees):

Baruj Benacerraf, M.D., Fabyan Professor and Chairman, Department of Pathology
David H. Katz, M.D., Assistant Professor of Pathology

2. Institution & address:

Harvard Medical School
25 Shattuck Street
Boston, Massachusetts 02115

3. Department(s) where research will be done or collaboration provided:

Department of Pathology

4. Short title of study:

Control of specific cellular and humoral immune responses to neoplastic and non-neoplastic tissues.

5. Proposed renewal date: July 1, 1973

6. How results to date have changed earlier specific research aims:

The initial results in the first six months of support by this Grant have been very gratifying and as shown in the Progress Report resulted in publications in press or to be submitted. Our results, therefore, have come up to our expectations and there is no reason to make any specific change in our stated research aims.

7. How results to date have changed earlier working hypothesis:

Our working hypotheses have not yet been fully tested but the results obtained so far greatly encourage us as to their validity.

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8. Any additional facilities now required? Describe briefly:

None

9. Any changes in personnel? Append biographical sketches of new key professional personnel:

No changes in personnel.

10. Append outline of experimental protocol for ensuing year.

11. List publications or papers in press resulting from this or closely related work. (append reprints or manuscripts not previously sent).

Katz, D.H., Order, S. W., Graves, M. and Benacerraf, B.: Purification of Hodgkin's Disease Tumor-Associated Antigens. In Press. Proceedings of the National Academy of Sciences.

Martz, E. and Benacerraf, B.: Inhibition of Immune Cell-Mediated Killing by Heparin. To be submitted to the Journal of Experimental Medicine.

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12. Summary progress report (append in standard form as separate document, unless recently submitted).

Source: https://www.industrydocuments.ucsf.edu/docs/[illegible]

13. Budget for the coming year:

A. Salaries (give names or state "to be recruited")

Professional (give % time of investigator(s) even if no salary requested)	% time	Amount
Baruj Benacerraf, M.D.	20%	-
David H. Katz, M.D.	40%	-
Technical		
M. Moran	100%	9,079.
M. Varney	50%	3,969.
M. Blanchard	100%	9,025.
W. Washington	40%	2,383.

Sub-Total for A 24,456.

B. Consumable supplies (by major categories)

Radioisotopes and chemicals	4,000.
Tissue culture media and materials	7,000.
Glassware and general supplies	2,000.

Sub-Total for B 13,000.

C. Other expenses (itemize)

Animals and animal care	16,544.

Sub-Total for C 16,544.

Running Total of A + B + C 54,000.

D. Permanent equipment (itemize)

Sub-Total for D

E. Indirect costs (15% of A+B+C) E 8,100.

Total request 62,100.

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14. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Studies on Experimental Cancer Immunology	National Cancer Institute National Institutes of Health - Pending	$497,506.	June 1, 1973 - May 31, 1974

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Checks payable to

-vard Medical School

Mailing address for check:

Business Office, Harvard Medical School

25 Shattuck St., Boston, Mass. 02115

Principal Investigator

Typed Name Baruj Benacerraf, M.D.

Signature Baruj Benacerraf Date 1/22/73

Telephone 617 (Area Code) 734-3300 (Number) 331 (Extension)

Responsible officer of institution

Typed Name Henry C. Meadow, Executive Secretary

Title Committee on Research and Development

Signature Henry C. Meadow Date 1/24/73

Telephone 617 (Area Code) 734-3300 (Number) 441 (Extension)

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10. Outline of Experimental Protocol for Ensuing Year.

1. Non-specific Enhancement of Anti-Tumor Immunity in Different Species and Experimental Tumor Systems.

The model of guinea pig L_2C leukemia employed in our original studies was chosen because of the immense challenge posed by the biological characteristics of this tumor. Thus, the rapid proliferation and disseminative nature leading to death at a relatively early and uniform time after administration makes successful immunotherapy a particularly difficult problem. This is further complicated by the fact that L_2C cells readily enter the protective environs of the central nervous system. Our significant success against such odds indicates, we believe, that such a model may be even more therapeutically efficacious in other tumor systems. We plan, therefore, to study the capacity of induced GVH reactions to enhance specific protective tumor immunity against a variety of experimental tumors, both in solid and ascitic form, in mice and rats and perhaps, hamsters. Moreover, we will adapt the model to studies of viral-induced leukemias in mice and hamsters.

In order to utilize this approach on a sound basis in species other than the guinea pig, it is essential to clearly establish appropriate conditions for induction of GVH reactions, of the type desired, in the species to be used. This is so because the magnitude of the GVH, among other less well-defined parameters, determines to a large extent the nature of the ultimate effect on the immune system of the host. Thus, although out initial observations in studies of antibody responses to hapten-carrier conjugates were concerned primarily with enhancing influences of the GVH on immune responses, further detailed analysis of the phenomenon illustrated clearly that a transient GVH reaction can suppress, with equal effectiveness, the immune system (1-3). Briefly, the most important obvious parameters in determining whether enhancement or suppression of immunity will result are those relating to 1) magnitude of the GVH, and 2) time of induction of the GVH with respect to presentation of the antigen(s) in question (1).

This point, and the importance of it, are well-exemplified by our experience over the past 1 1/2 years with the allogeneic effect in inbred mice. Studies were undertaken to determine optimal conditions for the allogeneic effect on antibody responses in mice as a prelude to utilizing this approach to enhance anti-tumor immunity in this species. However, what began as a reasonably straightforward pilot study, based on our previous knowledge of the system in guinea pigs, developed into a 1 1/2 year-long full-time research project. This resulted from the unforeseen complexities existing in inbred mice concerning major and minor histocompatibility differences, kinetic differences in optimal GVH reactivity, and perhaps also inherent genetic differences in immune system capabilities. Nevertheless, the conditions have now been developed, and carefully analyzed, for induction of appropriate GVH reactivity in a variety of mouse parental F_1-hybrid and congenic-resistant strains (1-4).

Having sufficient experience with the allogeneic effect model in mice, insofar as antibody responses are concerned, we are now able to begin to apply the appropriate conditions to studies of anti-tumor protection in this species. Toward this end, we are currently in the process of maintaining by serial transplantation several tumor lines in our own colony. These include the following tumors:

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1) Spontaneous mammary gland adenocarcinoma H2712 - C_3H host
2) Pleomorphic-cell sarcoma S37 (spontaneous mammary gland) - DBA/1 host
3) Anaplastic carcinoma dbrB (spontaneous mammary gland) - DBA/1 host
4) Methylcholanthrene-induced fibrosarcoma SaD_2 - DBA/a host

In order to standardize the system for meaningful tumor growth and survival statistics, we have been establishing dose response data utilizing freshly prepared viable single cell suspensions of tumor cells for the various lines. This will enable us to carry out experiments utilizing precise, uniformly lethal cell dose inocula for highly accurate comparisons of survival data between experimental and control groups. Over the next several months we will expand our mouse tumor lines to other available solid tumors and certain of the murine leukemias.

Our immediate plans are to begin studies of this type also in inbred rats. We are just beginning to analyze the pertinent characteristics of the allogeneic effect on antibody responses to defined antigens in rats, and to collect and serially transplant a number of transplantable rat tumor lines listed below:

1) CSE, fibrosarcoma (nickel sulfide iduced) - Fischer 344/CRBL host
2) RT-6 Cloned C_2 Glial (astrocytoma) tumor (N-nitroso-methylurea-induced) Wistar host
3) NT/W449 adencarcinoma (DES-induced) - W/Fu (female) host
4) NS 105 rhabdomyosarcoma (Nickel sulfide-induced) - Fischer 344 host
5) 13762 NM mammary adenocarcinoma (DMBA-induced) - Fischer males

The experimental plan in mice and rats will basically follow the design of the guinea pig L_2C leukemia studies. Thus, the GVH reaction, of appropriate magnitude, will be induced at varying times before, simultaneous with, or after inoculation of a lethal tumor cell inoculum and tumor growth characteristics and survival times will be compared statistically to controls. We assume that the optimal time interval of the allogeneic effect will have to be individually determined for each tumor and strain of mouse or rat involved. Once these basic parameters have been established, the systems can then be analyzed for answers to certain questions discussed in the following section.

2. Studies on the Effector Mechanisms in Host Anti-Tumor Immunity.

The studies on the protective effect of the GVH reaction on L_2C leukemia guinea pigs clearly demonstrated the participation of host immune mechanisms in the phenomenon but fell short of clearly delineating the nature of the host response. Nevertheless, certain indirect evidence derives from the guinea pig model which will be of great value when approaching the problem in inbred mice and rats.

For example, a crucial temporal relationship was found to exist between the transfer of allogeneic cells and the administration of the lethal L_2C leukemia inoculum. Thus, highly significant protection was observed in guinea pigs which received the allogeneic lymphoid cells 1, 3 or 6 days prior to lethal leukemic challenge. The protective effect was diminished somewhat in the cases where 13 days elapsed between cell transfer and leukemic challenge. In sharp contrast, transfer of allogeneic cells 21 days prior to leukemic challenge was completely ineffective in prolonging survival times, and when allogeneic cells were transferred immediately prior to challenge, only slightly prolonged survival times were observed.

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When considering which cells of the host may be primarily responsible for the anti-leukemic effect, there are essentially but two realistic possibilities: 1) the primary phagocyte cells of the RES, i.e., macrophages, and 2) the immunocompetent lymphocyte. It has been well established by others working with mice and rats that during the third week following induction of the GVH reaction in non-irradiated F_1 recipients of parental donor cells, there is a significant increase in resistance to bacterial infection (5,6). This has been attributed to heightened activation of the host macrophage population which has been shown to occur by the second or third week of the GVH response (7). It might reasonably be expected that sufficient numbers of such highly activated cells in the RES of the host could exert a non-specific anti-tumor cell effect. Indeed, Medzihradsky, working with a transplantable tumor in rats, interpreted a modest anti-tumor effect he observed in this way (8). In his system, prolonged survival was attained in F_1 recipients of parental cells transferred 14 days prior to tumor inoculation. Shorter or longer intervals between cell transfer and tumor challenge were ineffective. He suggested that the proliferation of host RES cells during the GVH reaction was responsible for this effect. In contrast, optimal anti-leukemic effects in our guinea pig system occurred much earlier in the course of the GVH response. The diminished protection observed in guinea pigs challenged with L_2C cells 13 days after allogeneic cells transfer and the failure to obtain anti-leukemic effects 21 days after transfer would argue against a primary role for hyperactive macrophages in this phenomenon. Nevertheless, a major role for these and other cells of the RES could not be excluded and lend themselves better for study in mice.

These results are, however, enlightening in the context of cellular kinetics in this phenomenon in that the transferred donor cells, themselves fully immunocompetent and thereby capable of reacting specifically against host tissues, were transplanted into a foreign environment consisting of similarly immunocompetent host lymphoid cells which not only possessed full capacity to reject the donor cells but maintained a decided advantage in numbers as well. In this circumstance we may assume, therfore, that essentially all allogeneic donor cells were rejected by the recipient in relatively short order. Assuming the 6 day interval between cell transfer and L_2C challenge to be optimal, it would seem very unlikely that sufficient numbers of donor cells, if any at all, were present in a fully functional state with the capacity to directly reject the inoculated L_2C cells. Employing the same reasoning in reverse, on the other hand, would make it a reasonable argument that in the cases of the 1-day or even, perhaps, the 3-day interval recipients, sufficient numbers of viable functional donor cells may exist to recognize and reject the foreign leukemic cells in the absence of active participation of host cells. However, if that were the case, the same effect should be true when allogeneic cells are transferred immediately prior to leukemic challenge, and this was not observed. The more appealing, and, perhaps, more likely explanation is that the critical cellular events in the expression of the protective phenomenon are ultimately performed by the host cell population which has been highly stimulated, in an as yet undefined manner, by the GVH reaction.

Evidence that the ultimate role is, in fact, performed by host cells was obtained in the guinea pig system (9). Hence, we demonstrated that protection against L_2C leukemia in this system requires the development of the GVH reaction in lymphoid organs of the host as evidenced by the failure to observe such protection in strain 2 recipients of semi-syngeneic $(2 \times 13)F_1$ hybrid donor cells. The ineffectiveness of $(2 \times 13)F_1$ lymphoid cells in affording anti-leukemia protection demonstrates that more than just antigenic stimulation of host cells is involved in the mediation of this effect. Furthermore, we found that passive

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transfer of parental strain 2 or strain 13 lymphoid cells into (2 x 13)F_1 recipients, which are genetically incapable of rejecting the parental donor cells, protects such recipients against the L_2C leukemia. This latter finding clearly demonstrated that the phenomenon depends solely on the existence of a GVH reaction and does not require the concomitant development of a host rejection response against donor lymphoid cells. The fact that parental strain 2 lymphoid cells were equivalent to strain 13 lymphoid cells in affording protection against leukemia in (2 x 13)F_1 recipients definitively excluded the possibility that donor cells directly reject the L_2C leukemia on the basis of an immune response directed at strain 2 histocompatibility antigens present of L_2C cells.

A major question, as yet unanswered definitively, concerns which subpopulation of host cells plays the primary role. As discussed above, we have considered the possible role of non-specific macrophages of the host RES. Studies on GVH reactions in mice and rats have established that during the second or third week of such reactions, a marked stimulation of RES function occurs (11). Since optimal protection in our guinea pig model occurs when leukemic challenge is made within the first week after induction of the GVH reaction, this may argue against hyperactive macrophages as the primary effector of the host. However, this argument is valid only if the stimulation of RES function during the GVH response in guinea pigs is similar in timing to that which has been demonstrated in other rodents. Since we are not aware of any definitive data on this point, such a role for macrophages in anti-leukemia protection has not been excluded. This makes an analysis of the critical time interval for anti-tumor protection by GVH in mice and rats absolutely essential.

It is more appealing, nonetheless, and perhaps more likely that immunocompetent lymphocytes of the host are the primary effector cells in this system. A suggestion of this derives from the demonstration in leukemic guinea pigs of persistent tumor immunity in 1/2 of the allogeneic cell recipients subjected to a second leukemic challenge (11). There are several possible mechanisms by which host lymphocytes may exert such a protective effect. One possibility is the production of specific anti-L_2C antibodies of a protective type which could effect destruction and/or removal of the leukemic cells. Our failure to detect specific protective antibodies by in vivo serum transfer and by several in vitro techniques argues against a role for such molecules in this phenomenon (9). However, these studies by no means rule out the production of such antibodies which may be bound to L_2C cells in host tissues, and therefore, not present in serum, or their presence in serum in quantities too small for detection by the techniques employed. More importantly, it is very conceivable that while anti-leukemia protection in guinea pigs may not involve specific antibodies, the converse may turn out to be the case in such systems in mice, rats or even humans. Alternatively, it will be of invaluable importance to determine whether an effect, perhaps suppressive, on synthesis of enhancing antibodies by the GVH can be demonstrated. This should be considerably easier to assess in tumor systems in mice and rats known to be complicated by such antibodies.

Another alternative possibility, also easier assessed in mice and rats utilizing standard in vitro procedures, is the production, by host lymphocytes, of non-specific cytotoxic factors as a result of the immunological attack of grafted donor cells against host tissues. Recently, such cytotoxic factors produced by host cells following induction of the GVH reaction have been demonstrated to destroy tumor cells (11). The operation of such a mechanism would not preclude the possibility that tumor-specific immuntiy may develop during the period of non-specific destruction of the tumor inoculum.

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Finally, we have favored the possibility that the anti-tumor effect in this system is a manifestation of specific cellular immunity. In a wide variety of experimental and human tumors, it appears that some degree of tumor-specific cellular immunity may exist despite the fact that the tumor continues to actively proliferate (12). The induction of a GVH reaction may serve as a potent stimulus for the initiation of marked augmentation of a specific cell-mediated immune response directed against the tumor. The observation in the L_2C leukemia system that persistent tumor immunity against leukemia existed in 1/2 of the long-term surviving allogeneic cell recipients which were subjected to rechallenge supports this hypothesis (10). Nevertheless, it was not possible to prove in that system that specific cellular immunity operates as the primary effector mechanism in this system and further investigation on this point will be absolute necessary.

A crucial point of focus in the proposed studies will be to determine whether or not specific cell-mediated anti-tumor immunity has been induced, or augmented, by the allogeneic effect in the tumor-bearing host. This can be most readily studied by adoptive cell transfer experiments in non-irradiated syngeneic recipients in both mice and rats. Studies utilizing *in vitro* techniques for evaluating specific cell-mediated immunity will also be performed toward this end. Since specific antisera are now available for selective removal of T or B lymphocytes in both of these species, it will be possible to derive a definitive answer to the question concerning the predominant effector mechanisms of anti-tumor protection in the allogeneic effect.

3. Comparative Studies on Other Non-Specific Stimulants of the Immune System.

Although the major emphasis of this proposal is to analyze in detail the efficacy of the allogeneic effect for augmentation of tumor-specific immunity, firm conclusions in this regard will necessitate full comparative evaluation of other methods known to heighten, non-specifically, the immune system in general. Once the various models proposed in the aforementioned project are established, it will be desirable and readily possible to perform such studies. Thus, the potency and comparative effectiveness of stimuli such as the T cell mitogens, concanavalin A and *E. coli* lipopolysaccharide (endotoxin), infectious agents such as Listeria monocytogenes and derivatives thereof (i.e., cell extracts), other non-specific adjuvants such as polyadenylic:polyuridylic acid (Poly A:U), and beryllium salt, and macrophage-activating substances can be thoroughly evaluated. Effects of the above on stimulation of tumor immunity will be assessed for cell-mediated as well as humoral (enhancing vs cytotoxic antibodies) components both *in vivo* and *in vitro*, and, of course, the respective roles of lymphocytes and macrophages will be analyzed.

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Source: https://www.industrydocuments.ucsf.edu/docs/jzvf0000

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Source: https://www.industrydocuments.ucsf.edu/docs/fzyl0000

Mechanism of Specific Cytolysis of Malignant Target Cells by Immune Thymus-Derived Lymphocytes.

There are a number of *in vitro* models for the several mechanisms involved in graft or tumor rejection *in vivo* (1,2). These involve cytotoxicity to target cells mediated by effector cells from lymphoid tissues, as first described by Govaerts (3). Such cell-mediated toxicity can be divided into two categories, according to the type of effector cells responsible: the first category includes cytotoxicity effected by non-thymus-derived cells, such as "B" lymphocytes (4,5) or monocyte-macrophage cells (6,7,8). The second concerns cell-mediated killing effected directly by thymus-derived lymphocytes. The first type of killing most likely depends upon antibodies (1,5) which may be released locally by antibody-producing "B" lymphocytes (plasma cells) in some *in vitro* systems. Since tumor and graft rejection is transferred by cells but not by serum (9) we feel that cytotoxicity of the second type, or related activities of the thymus-derived effector cells, must be crucial in specific tumor or graft rejection. Therefore, we shall be concerned primarily with this second phenomenon.

Using the mouse DBA/2 P815 mastocytoma as the target for allo-immunized killers, it has been shown that thymus cells can give rise to killer cells (10) and that cells bearing the theta antigen are necessary for this cytolytic activity (11). Thymus-derived lymphocytes can probably kill without "help" from bone marrow-derived cells, since attempts to remove the latter have not diminished killing activity (12,13,14). Specific killer cells can be produced *in vitro* by culturing lymphoid cells with antigen-bearing cells (15-18) and killers produced in this way are the products of cell division (15,19). The killer cell bears antigen recognition sites which allow it to be specifically removed from a lymphoid population by adhesion to antigen-bearing fibroblast monolayers, and subsequently recovered from the monolayer (20,21). Covering up the antigenic sites on the target cell with alloantisera inhibits lysis (22,23). Killing by these effector cells is relatively rapid: a majority of the target population can be lysed in less than 1 hour (12,13). In this system, non-specific ("bystander") target cells, when mixed among immune lymphocytes and specific targets, are relatively unharmed by the closely adjacent killing process (13,15, 24-26); the killers themselves are not lysed (27,28) and are able to kill a second population of targets at the same or greater rate than the first (12,28). Target cell killing progresses in medium containing heat-inactivated serum, and addition of exogeneous complement does not affect the rate of cytolysis (24,29,30). The killing process, however, requires some heat-stable non-dialyzable serum components (13) as well as calcium or magnesium ions (31).

In spite of the advances just described, much remains to be elucidated about specific target cell lysis by immune thymus-derived lymphocytes. The questions can be grouped in three categories:

I. What is the developmental origin, the pathway of differentiation, and the fate of the thymus-derived killer lymphocyte?

   a) What is the immediate precursor of the effector cell? How much proliferation is required? And where does this proliferation and differentiation take place *in vivo*?

   b) What is the lifetime of the effector cell?

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c) Is the effector cell in a terminal state of differentiation?

d) Are there killer memory cells? Is there a secondary killer response?

e) Are there more than one type or state of thymus-derived killer lymphocytes (26,32)?

f) What is the relationship between the killer cell and cells effecting other thymus-derived functions (such as release of MIF, graft vs host reactions, mixed lymphocyte reactions)?

II. What is the mechanism by which the sensitized thymus-derived lymphocyte kills the target cell? Even less is known about this mechanism than about the nature of the killer cell.

a) How long does the killing of a single target cell require? Is this time constant or variable? If variable, does it depend on the state of the killer or the target? Is the difference in killing rate for different targets bearing the same alloantigens (12) a function of the speed of each killing event, or the rate at which the killer can move from one target cell to the next?

b) Does the killing of several targets by one killer (12,28) occur in sequence or in parallel?

c) Do cell membrane movements or killer cell locomotion play any role in killing?

d) What are the steps in killing, what is their sequence, and how long does each take?

e) What proteins or other substances must be synthesized (23) after the killer is confronted with specific antigen, and what is their role?

f) Do the soluble cytotoxins found in culture supernatants (33,34) play a role in cell-mediated killing?

g) What serum factors are required and what is their role? Are they equivalent to those required for survival, movement or DNA synthesis of fibroblasts (35)?

h) Are divalent cations, serum factors, and 37°C temperature required throughout the killing process or only at certain critical steps, perhaps each with different requirements?

i) Is osmotic disequilibrium across the cell membrane necessary for completion of lysis, as it is for complement mediated lysis (36)?

j) What accounts for the great strength of the adhesion between killer and target?

k) What is the ultrastructural morphology of the killer, and its interaction with the target at various stages during killing?

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III. What is the role of sensitized thymus-derived killer lymphocytes in tumor or graft rejection in vivo? This is the most difficult question to resolve, since it depends on a better understanding of I and II above. We must learn how many of each kind of effector cell are present in the tumor tissue during the stages of rejection (which will require enumerative assays for each functional class of cell), how and why they got there, and their relative contributions to death of the tumor tissue. Ultimately, it should be possible to manipulate the effector cell system so as to potentiate damage to tumor tissue.

Experimental Design

Initially, we plan to focus our research efforts largely on the mechanism of specific target cell killing by sensitized thymus-derived lymphocytes (category II above). The killing is undoubtedly a multistep process (30) which depends initially upon the interaction of antigen on the target cell membrane with specific lymphocyte receptors.

Experimentation is in progress in our laboratory to attempt to resolve this sequence of steps through studies utilizing inhibitors. We have found (37) that heparin (previously known to interfere with several forms of cellular immunity [38-40]) and other highly charged polyanions retard a rapid, early killer-target interaction necessary for killing. Once this interaction has occurred, it cannot be reversed by subsequently added heparin, and lysis proceeds to completion at the normal rate (see progress report).

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Source: https://www.industrydocuments.ucsf.edu/docs/fzyd0000

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